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FMRFamide related peptide ligands activate the *Caenorhabditis elegans* orphan GPCR Y59H11AL.1

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ABSTRACT

G-protein coupled receptors (GPCRs) are ancient molecules that can sense environmental and physiological signals. Currently, the majority of the predicted *Caenorhabditis elegans* GPCRs are orphan. Here, we describe the characterization of such an orphan *C. elegans* GPCR, which is categorized in the tachykinin-like group of receptors. Since the *C. elegans* genome predicts only one tachykinin-like peptide (SFDRMGGTEFGLM), which could not activate the receptor, we hypothesized that one or some of the numerous FMRFamide related peptides (FaRPs) could be the cognate ligands for this receptor. This hypothesis was based on the suggestion that RFamides may be ancestral neuropeptides, from which a lot of the amidated neuropeptides, including tachykinins, derived. Indeed, we found that the orphan receptor encoded by the Y59H11AL.1 gene is activated by several *C. elegans* neuropeptides, including SPMERSAMVRFamide. These peptides activate the receptor in a concentration-dependent way.

1. Introduction

Caenorhabditis elegans flp-genes encode a family of peptides ending in RFamide that belong to the family of FMRFamide related peptides (FaRPs). To date, about 70 FaRPs have been predicted from the *C. elegans* genome, encoded by 28 *flp*-genes [13,10,6,16]. These neuropeptides serve as important neurotransmitters or neuromodulators in the nervous system. To find the exact function of all these peptides, it is extremely important to identify all the neuropeptide-receptor pairs. The *C. elegans* genome codes for about 1000 GPCRs, 54 of which could code for neuropeptide receptors. The pairing of the putative receptors and their ligands, however, remains an arduous task, despite the publication of the *C. elegans* genome more than 6 years ago [29]. In the past two years, only four *C. elegans* neuropeptide GPCRs have been paired with their ligand [11,12,18,17], in contrast to the numerous vertebrate GPCRs, for which the cognate ligands have been identified. Nevertheless, as *C. elegans* is a model organism for studying several signaling pathways at the organismal level in a multicellular organism, it is important to characterize the membrane receptors within this nematode.

In this study, we describe the characterization of an orphan neuropeptide receptor encoded by gene Y59H11AL.1 (GenBank accession no. AC024840). This receptor is classified in the group of tachykinin-like receptors [8]. Mammalian neurokinin and invertebrate tachykinin receptors are known to be conserved during evolution (peptide-receptor co-evolution) [30] and are known to play important functions in the control of motor activities. However, the Y59H11AL.1 receptor is also

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very closely related to the *Drosophila* neuropeptide Y-like receptor (CG5811 – NepYr) (www.wormbase.org).

We developed cells (HEK-293 cells) transiently expressing the Y59H11AL.1 receptor and screened 68 synthetic FaRPs, mined from the *C. elegans* genome [16,20,13], for a calcium response using a fluorescence assay. We show that several peptides, including (SPMERSAMVRFamide) ending in MVRFamide activate the cloned receptor in a concentration-dependent way.

2. Materials and methods

2.1. GPCR cloning

The ORF of the predicted Y59H11AL.1 gene was amplified by PCR performed on the cDNA (SuperScript First-Strand Synthesis System for RT-PCR, Invitrogen, The Netherlands), synthetized from mRNA (QuickPrep *micro* mRNA Purification Kit, Roche, IN, USA) of whole nematodes. Specific oligonucleotide PCR primers with incorporated restriction sites (Eurogentec, Belgium) were used (forward primer 5′-CAG GAT CCG CCA CCA TGG ACG AAG GAG GGG GTA TTG G-3′; reverse primer 5′-CAT CTA GAG GTC TTC TAT AGC TTT CCA CTC AAG-3′). The Advantage 2 PCR kit (Clontech, USA) was used under the following PCR conditions: the reaction mixture was first denaturated at 95 °C for 180 s, then subjected to 30 cycles of 94 °C for 60 s, 68 °C for 60 s, 68 °C for 180 s and an extra extension step of 3 min at 68 °C. The obtained PCR product was first cloned in the pCR2.1-TOPO vector using the TOPO-TA Cloning kit (Invitrogen, The Netherlands). After selection and automatic sequencing (310 Genetic Analyzer, Applied Biosystems, UK), the receptor was directionally cloned into the pcDNA3 mammalian expression vector (Invitrogen).

2.2. Generation of cells transiently expressing C. elegans Y59H11AL.1 receptor

Human embryonic kidney (HEK293) cells were cultured at 37 °C in a humified atmosphere of 5% CO_2 in DMEM medium (BioWhittaker, Belgium), supplemented with 10% heat inactivated horse serum, non-essential amino acids, 100 units/ml penicillin and 100 μg/ml streptomycin. The Y59H11AL.1/pcDNA3 construct was transfected into the HEK293 cells using FuGene 6 (Roche, IN, USA). The promiscuous G-protein $G_{\alpha 16}$ was cotransfected with the receptor construct. A total amount of 15.6 μg plasmid DNA, containing the receptor construct and $G_{\alpha 16}$ construct, was mixed with 93.6 μl FuGene 6 in 1406.3 μl serum free DMEM medium and incubated at room temperature for 15 min. The mixture was then applied to the cell flask (150 cm^2) containing 8 ml supplemented DMEM medium and the cells were incubated at 37 °C for 24 h.

2.3. Fluorescence assay

Cells were detached 24 h after transfection and subsequently plated out in 96-well plates at approximately 70% confluence. 48 h after transfection, the cells were loaded with fluorophore, Fluo-4-AM (Molecular probes, The Netherlands) for 1 h, after which excess fluorophore was washed away with HBSS* buffer (HBSS, supplemented with 5 mM $CaCl_2$ and 10 mM Hepes). Excitation of the fluorophore was achieved at 488 nm. Fifty μl of different concentrations of the synthetic peptides, diluted in HBSS* buffer, were transferred from the compound plate to the 96-well plate containing the HEK-cells. The calcium response was measured for 2 min at 525 nm using the FLEXStation (Molecular Devices) at 37 °C. Data were analyzed using Softmax Pro (Molecular Devices).

In the temperature shift experiments, the cells were placed at 28 °C 24 h after transfection, after the cells were plated out in the 96-well plates. The measurements were also done at 28 °C in these experiments. All the experiments were also conducted with cells transfected with the pcDNA vector without receptor insert, as a negative control. Cell viability was tested with bradykinin, activating an endogenous HEK cell receptor. As a transfection control, we also transfected cells with a receptor for which the ligand is known (*Drosophila* short neuropeptide F receptor).

2.4. Peptide synthesis

All peptides were either custom-synthesized (Invitrogen) or made in house using conventional Fmoc chemistry.

3. Results

3.1. Cloning of the full-length cDNA of C. elegans Y59H11AL.1 receptor

PCR amplification of the cDNA with oligonucleotide primers specific for the predicted ORF of Y59H11AL.1 produced a single product of approximately 1300 bp (data not shown). Sequence determination of the TA-cloned PCR-product revealed a DNA insert of 1284 bp, corresponding to the sequence of the six predicted exons of gene Y59H11AL.1 (www.wormbase.org). The deduced protein encoded by the ORF of the Y59H11AL.1 gene is 427 amino acids long (Fig. 1). Analysis by the TMHMM program (www.cbs.dtu.dk/services/TMHMM-2.0/) revealed that this protein is predicted to have seven transmembrane domains along with the intracellular and extracellular loops, consistent with the known structure of G-protein coupled receptors. The N-terminal extracellular region exhibits one O-glycosylation (www.cbs.dtu.dk/services/NetOGlyc/) and no N-glycosylation sites (www.cbs.dtu.dk/services/NetNGlyc/). The intracellular C-terminal region exhibits 12 possible phosphorylation sites (www.cbs.dtu.dk/services/NetPhos/) (Fig. 1).

3.2. Fluorescence assay

Following successful cloning of the receptor and the generation of cells transiently expressing the Y59H11AL.1 gene, the cells were challenged with different synthetic peptides predicted in the *C. elegans* genome, including FaRPs (encoded by *flp* genes) and NLPs (encoded by *nlp* genes). A total of 68 peptides were used to screen the receptor (Table 1). All the peptides that were able to elicit a calcium response are depicted in Table 2. One of the active peptides, SPMERSAMVRFamide, was able to elicit a clear calcium response with an EC_{50} value smaller than 1.1 ± 0.1 μM ($n = 3$) (Fig. 2). Other active peptides

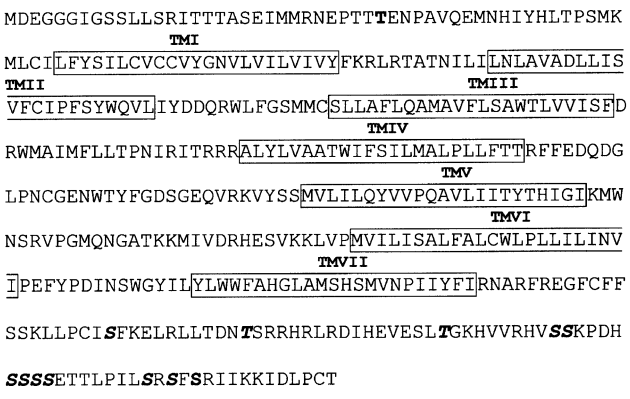

Fig. 1 – Amino acid sequence of the open reading frame of the Y59H11AL.1 gene. The seven transmembrane domains are framed, the intracellular phosphorylation sites are depicted in *bold*, *italic* and the extracellular glycosylation site is depicted in bold.

are KPNFMRYamide, encoded by *flp*-1, TPMQRSSMVRFamide, SPMQRSSMVRFamide and SPMDRSKMVRFamide, encoded by *flp*-7, KPSFVRFamide, encoded by *flp*-9, ASGGMRNALVRFamide, AMRNAVLRFamide and NGAPQPFVRFamide, encoded by *flp*-11, AADGAP LIRFamide, ASPSAPLIRFamide, ASSAPLIRFamide, SAAAPLIRFamide and SPSAVPLIRFamide, encoded by *flp*-13 and SPSA KWMRFamide, encoded by *flp*-22 (Table 2).

We also tested all peptides at a concentration of 10 μM in receptor expressing cells that were incubated at 28 °C for 40 h after transfection, since the EC_{50} value of the most active peptide is in the micromolar range, which is unusually high. EC_{50} values are commonly in nanomolar or even subnanomolar ranges for most vertebrate or *Drosophila*

Table 1 – Amino acid sequences of all 68 synthetic *C. elegans* neuropeptides (10^{-5} M) tested for a calcium response using the fluorescence assay

Gene	Peptide sequence
Neuropeptide-like proteins (*nlp* genes)	
nlp-1	MDANAFRMSFamide
nlp-2	SIALGRSGFRPamide
nlp-3	AVNPFLDSIamide
nlp-6	AAMRSFNMGFamide APKQMVFGFamide
nlp-7	pQADFDDPRMFTSSFamide LYLKQADFDDPRMFTSSFamide
nlp-10	AAIPFSGGMYamide
nlp-11	HISPSYDVEIDAGNMRNLLDIamide SAPMASDYGNQFQMYNRLIDAamide SPAISPAYQFENAFGLSEALERAamide
nlp-12	DYRPLQFamide
nlp-13	pQPSYDRDIMSFamide SPVDYDRPIMAFamide
nlp-14	ALNSLDGAGFGFE
nlp-17	GSLSNMMRIamide
nlp-22	SIAIGRAGFRPamide
nlp-23	SMAIGRAGMRPamide AFAAGWNRamide
nlp-30	pQWGYGGYamide
FMRFamide-like peptides (*flp* genes)	
flp-1	SADPNFLRFamide AAADPNFLRFamide PNFLRFamide **KPNFMRYamide**
flp-2	SPREPIRFamide LRGEPIRFamide
flp-3	SPLGTMRFamide EAEEPLGTMRFamide ASEDALFGTMRFamide SAEPFGTMRFamide SADDSAPFGTMRFamide NPENDTPFGTMRFamide
flp-4	PTFIRFamide ASPSFIRFamide
flp-5	GAKFIRFamide
flp-6	KSAYMRFamide SAYMRFamide
flp-7	**SPMQRSSMVRFamide** **TPMQRSSMVRFamide** **SPMERSAMVRFamide** **SPMDRSKMVRFamide**
flp-9	**KPSFVRFamide**
flp-10	QPKARSGYIRFamide
flp-11	**AMRNALVRFamide** **ASGGMRNALVRFamide** **NGAPQPFVRFamide** SPLDEEDFAPESPLQamide
flp-12	RNKFEFIRFamide
flp-13	**AADGAPLIRFamide** APEASPLIRFamide **ASPSAPLIRFamide** **SPSAVPLIRFamide** **ASSAPLIRFamide** **SAAAPLIRFamide** AMDSPLIRFamide
flp-14	KHEYLRFamide
flp-15	RGPSGPLRFamide
flp-16	AQTFVRFamide
flp-18	SEVPGVLRFamide DVPGVLRFamide
flp-19	WANQVRFamide
flp-22	**SPSAKWMRFamide**
Peptides derived from newly identified neuropeptide precursors	
CE18432	EIVFHQISPIFFRFamide SLLDYRFamide
NM_064842.2	VPSAGDMMVRFamide
NP_741827	EFNADDLTLRFamide
NP_741828	GGAGEPLAFSPDMLSLRFamide

Peptides depicted in bold were able to activate the receptor.

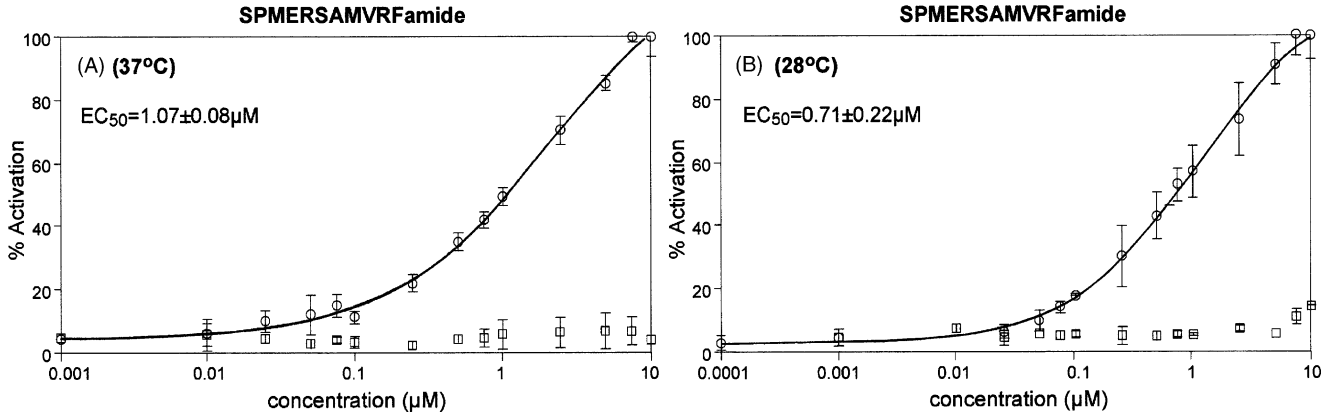

Fig. 2 – Dose response curve of the effect of SPMERSAMVRFamide on the *C. elegans* Y59H11AL.1 receptor expressed in HEK293 cells incubated at 37 °C (A) and 28 °C (B); Fluorescent responses of the cell line expressing the orphan receptor are expressed in % activation. Receptor responses (pcDNA/receptor) are represented by (○) and negative control responses (pcDNA3) are represented by (□). These are the collected data of 3 independent measurements. The vertical bars represent standard deviations. Data were processed using Softmax Pro software (Molecular Devices).

Table 2 – Synthetic peptides, amino acid sequences and activity as tested in various concentrations (10^{-5}–10^{-10} M) for a calcium response using the fluorescence assay using cells incubated at 37 °C

Peptide sequence	Gene	Activity (threshold in μM)
KPNFMRYa	*flp*-1	0.1
TPMQRSSMVRFa	*flp*-7	2.5
SPMQRSSMVRFa	*flp*-7	5
SPMERSAMVRFa	*flp*-7	0.025 ($EC_{50} < 1.1 \pm 0.1$)
SPMDRSKMVRFa	*flp*-7	5
KPSFVRFa	*flp*-9	5
ASGGMRNALVRFa	*flp*-11	2.5
AMRNALVRFa	*flp*-11	0.75
NGAPQPFVRFa	*flp*-11	1
AADGAPLIRFa	*flp*-13	5
ASPSAPLIRFa	*flp*-13	5
SPSAVPLIRFa	*flp*-13	2.5
ASSAPLIRFa	*flp*-13	5
SAAAPLIRFa	*flp*-13	5
SPSAKWMRFa	*flp*-22	1

Threshold values are given since a plateau is not reached at a concentration of 10 μM (EC_{50} values cannot be calculated).

concentration-response curves for receptor activation by peptides. We found that the same peptides activated the receptor in cells with or without the temperature shift.

The most active peptide, SPMERSAMVRFamide, was also tested at different concentrations using temperature-shifted cells (Fig. 2). We observed that the EC_{50} value using cells incubated at 28 °C ($EC_{50} < 0.7 \pm 0.2$ μM) ($n = 3$) was not significantly different from the value using cells that were incubated at 37 °C ($EC_{50} < 1.1 \pm 0.1$ μM) ($n = 3$).

4. Discussion

BLAST searches of the *C. elegans* database reveal over 1000 G-protein coupled receptors. Fifty-four of these receptors display similarities to vertebrate neuropeptide receptors and may include FMRFamide-like peptide (FLP) receptors [13] or neuropeptide-like peptide (NLP) receptors [20]. To date, only 4 *C. elegans* neuropeptide receptors have been identified, namely the FLP15 peptide receptor [12], the AF9 receptor [11], the VRFamide receptor 1 [18] and the FLP-2 receptors [17]. The orphan receptor encoded by gene Y59H11AL.1 belongs to the tachykinin-like receptor family [8]. Tachykinins (or neurokinins) belong to a large family of multifunctional brain/gut peptides that play a role in the central nervous system as well as in the peripheral nervous system and they are involved in sensory processing and in motor and intestinal motility [30]. Substance P was the first member of this family to be discovered as a factor that causes peripheral vasodilatation and stimulates intestinal muscle contraction [32]. All members of the vertebrate and a few members of the invertebrate family share the C-terminal motif FXGLMamide [30]. In invertebrates, the first sequence of a tachykinin was characterized in the mollusk *Eledone moschata* [4]. Locustatachykinins were the first insect tachykinins isolated from a CNS extract and they were found to stimulate muscle contractility like their vertebrate counterparts [28,27]. Insect tachykinins all share the conserved sequence motif $GFX_1GX_2Ramide$ [30]. The *C. elegans* genome only revealed one peptide, the last *nlp*-8 predicted peptide (SFDRMGGTEFGLM), that contains a C-terminal tripeptide of sequence identity with human substance P [20]. However, this peptide was one of the 68 peptide sequences tested and it was not able to activate the receptor, so it is likely that the ligands for the cloned tachykinin-like receptor will belong to another group of neuropeptides. Since the Y59H11L.1 receptor also displays homology to the *Drosophila* neuropeptide Y-like (NepY) receptor, we searched for possible ligands among the FaRPs, because in several invertebrates, NPY-like receptors are activated by FMRFamide related peptides [8]. These FaRPs are also considered to be very ancient peptides, from which a lot of other amidated peptides, including tachykinins, may have been derived [31,1].

Several peptides, including the most potent peptide SPMERSAMVRFamide, belonging to the FaRP family, were indeed able to clearly activate the cloned receptor in a concentration-dependent way. The EC_{50} value of SPMER-

SAMVRFamide, however, is in the micromolar range (0.71 μM), even after the temperature shift to 28 °C. The idea for this temperature shift came from Kubiak et al. [12], who found that a temperature shift to 28 °C was absolutely critical for the functional expression of their receptor in mammalian cells. Without this temperature lowering step, no significant peptide-invoked receptor activation could be detected in their assay. A similar pattern was found for the NPR-1 receptor [11]. Since the optimal temperatures for free living nematodes are 15–19 °C, it might be that *C. elegans* receptors need lower temperatures for proper folding and functional expression. Kubiak et al. [12] found EC_{50} values in the high nanomolar range (160–600 nM) for the FLP-15 receptor and the EC_{50} value for activation of the recently identified VRFamide receptor 1 by TPMQRSSMVRFamide [18] using cells incubated at 37 °C is in the micromolar range. These measured high EC_{50} values could be due to the heterologous nature of the cellular expression system. It could be that the *C. elegans* receptors need not only lower temperatures, but also different accessory proteins than those present in mammalian cells for proper folding and functional expression. This poor worm receptor-mammalian host compatibility could be responsible for the slow progress that is being made in deorphanizing the more than 50 orphan neuropeptide receptors left in the *C. elegans* genome.

The most active peptide identified in this study, can also activate the VRFamide receptor 1 as previously described [18] and the most potent ligand of the VRFamide receptor 1, TPMQRSSMVRFamide, can also activate the Y59H11AL.1 receptor. So, if the peptides also activate both receptors in vivo, they could be redundant, but the selectivity of both receptors for the peptides in vitro is different. AMRNALVRFamide and SPMQRSSMVRFamide are also peptides that can activate both receptors. However, both receptors are not related since the VRFamide receptor 1 is not aligned in the tachykinin receptor family and they only show 24% sequence similarity. Another peptide encoded by the same *flp*-7 precursor, SPMDRSKMVRFamide, can only activate the Y59H11AL.1 receptor. A full functional and pharmacological analysis will probably be necessary to better understand the nature of these findings. The studies of both these G-protein coupled receptors also indicate that peptides from different peptide precursors can activate a single receptor. This is different in the case of the FLP-15 and the FLP-2 receptors, that can only be activated, respectively by the *flp*-15 and *flp*-2 precursor derived peptides. The FLP-9, FLP-18 and FLP-21 peptides that are capable to activate the NPR-1 receptor [25], were not included in our peptide library.

The *C. elegans* genome contains at least 28 *flp*-genes that encode about 70 structurally different FaRPs [13,6,10,16]. The first known FaRP, FMRFamide, was isolated in 1977 as a cardioactive agent on the molluscan heart [24]. Since then, FaRPs have been found in the nervous system of animals representing all major phyla [22,26,33,23]. These peptides have been shown to have diverse functions in invertebrates such as cardioexcitation [5], control of muscle contraction [2,15] and neuromodulation [3]. In vertebrates, they have anti-opioid effects [19]. *C. elegans* FaRPs are expressed in at least 10% of the neurons, including motor, sensory and interneurons that are involved in movement, feeding, defecation and reproduction [12]. The *flp*-7 gene, which encodes the most potent Y59H11AL.1 receptor-activating peptide, SPMERSAMVRFamide, is expressed in all stages of development [21]. Deletion of the gene does not yield a specific phenotype [7,14]. RNAi analysis of 60 GPCRs by Keating et al. also did not reveal a phenotype for the Y59H11AL.1 receptor when tested in egg laying, brood size and locomotion assays [8]. This is surprising since the receptor is categorized as a tachykinin-like receptor. A possible explanation for these results is that the receptor is likely to be expressed in the neuronal cells of the brain. In *C. elegans*, 90% of neuronal cells are resistant to gene inactivation by RNAi [9]. The problem seems to be the systemic character of the method: most neurons do not take up the silencing trigger. An explanation of the wild type phenotype in worms with a deleted *flp*-7 gene, is the possible redundancy of one or more ligands of the receptor. Further experiments will have to prove these assumptions and will have to prove that the tachykinin-like function we propose indeed is conserved.

5. Conclusion

In conclusion, we cloned and characterized the *C. elegans* receptor encoded by gene Y59H11AL.1. This receptor was predicted to belong to the family of tachykinin-related receptors. However, the only predicted tachykinin-related peptide in *C. elegans* was not able to activate the receptor when expressed in HEK cells. Instead, several FMRFamide related peptides, including SPMERSAMVRFamide, can elicit a clear calcium response in a concentration-dependent way.

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